REMARKS

Status of the Claims

Claims 1, 6-9, and 12-18 are currently pending and under examination. Claims 2-5, 10, and 11 have been canceled without prejudice or disclaimer of the subject matter claimed therein. These claims are thought to be allowable for the reasons noted below and otherwise of record.

Amendments to the Claims

Claim 1 has been amended to recite the features of canceled claims 4 and 5. Claim 1 has been further amended to recite that the type III AFP have at least 80% identity with SEQ ID NO: 1. Representative support is found at page 9, lines 10-21 of the specification.

Claims 14-18 are new. Representative support for the new claims can be found in claims 1, 4, 5, 9, and at page 9, lines 10-21 of the specification.

The amendments to the claims do not add prohibited new matter.

Claim Objections

Claims 6 and 7 have been objected to for reciting an abbreviation without reciting the entire phrase for which the abbreviation is used. Claim 1, as amended, recites "protein mannosyl transferase" and further recites its corresponding acronym "pmt", which is referenced in claims 6 and 7. This is thought to obviate the basis for the Examiner's objection. Accordingly, withdrawal of the objection is respectfully requested.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 1-8 and 12 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly failing to particularly point out and distinctly claim the subject matter regarded as the invention.

The Examiner referred to this rejection under the heading of 35 U.S.C. § 112, first paragraph. However, the Applicants note that the text of 35 U.S.C. § 112, second paragraph, is quoted and the Examiner's rejection under 35 U.S.C. § 112 is apparently based on the second paragraph. For the purposes of this response only, Applicants assume the Examiner intended the

rejection to concern the second paragraph and have responded accordingly.

The Examiner alleged that the term "type III antifreeze protein" is unclear in the art. Claim 1, as amended, recites that the type III antifreeze protein has at least 80% sequence identity with SEQ ID NO: 1. Accordingly, it is believed that the type III AFP as now claimed is clear and distinct with respect to the intended scope of the invention. One skilled in the art would appreciate that a type III AFP with at least 80% identity to SEQ ID NO: 1 is a distinct type III AFP in its composition. It is therefore respectfully requested that the Examiner's rejection under 35 U.S.C. § 112, second paragraph, be withdrawn.

Rejection Under 35 U.S.C. § 112, First Paragraph

A. Claims 1-9, 12, and 13 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

The Examiner alleged that the claims contain subject matter not described in the invention sufficiently to convey possession of the claimed invention at the time the application was filed. The Examiner alleged that the scope of the term "type III AFP" encompasses a broad spectrum of AFP proteins from various species that vary widely in overall protein size. Without acquiescing to the merits of the rejection, Applicants have amended the claims to recite that the type III AFP have at least 80% identity to SEQ ID NO: 1.

The Examiner also alleged that the specification does not provide support for all types of fungus host cell deficient in protein glycosylation. Applicants have amended the claims to more specifically recite that the fungal host cell is a yeast cell deficient in pmt1 and/or pmt2. Yeast cells belong to the *Saccharomycotina* subphlyum of the fungi phylum *Ascomycota*. It is respectfully submitted that it is well known in the art that the provided species example in the specification of *Saccharomyces cerevisae* is a model organism for yeast. Furthermore, the specification details identification of pmt homologues in the yeast *Kluyveromyces lactis* based on homology to *Saccharomyces cerevisae* (*see* page 17, lines 10-29 of the specification). It is also noted that others in the art state pmt proteins are evolutionarily conserved and have been identified in other yeasts (*see* attached reference of Strahl-Bolsinger *et al.*, J. Biol. Chém. 274: 9068-9075, 1999, at abstract and introduction). It is therefore respectfully submitted that the claimed invention is enabled by the specification for yeast dficient in protein glycosylation.

Accordingly, it is believed the claims, as amended, comply with the written description requirement. Applicants therefore respectfully request that the Examiner's rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

B. Claims 1-9, 12, and 13 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to enable the claimed invention. The Examiner alleged that the specification fails to enable producing any type III AFP polypeptide using any fungal host deficient in protein glycosylation.

The Examiner alleged that the specification does not enable a method of producing any type III AFP in any type of fungal host cell deficient for glycosylation. The claims, as amended, recite that the fungal host cell is a yeast, and that the host is deficient in pmt1 and/or pmt2. The claims, as amended, further recite that the type III AFP have at least 80% identity with SEQ ID NO: 1.

As is mentioned above, the specification points out that pmt proteins in other yeast are known in the art or are readily identified. Furthermore, the Examiner acknowledged that the specification discloses inactivated pmt1 and/or pmt2. Accordingly, it is believed that the claims, as amended, directed to pmt1 and/or pmt2 deficient yeast are enabled by the specification.

Furthermore, the claims as amended are directed to a type III AFP with at least 80% homology to SEQ ID NO: 1. The Applicants' invention as thus claimed is fully enabled. It is therefore respectfully requested that the Examiner's rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

Rejection Under 35 U.S.C. § 103(a)

Claims 1-9, 12, and 13 have been rejected under 35 U.S.C. § 103(a) as allegedly being obvious under Chapman *et al.* (WO 97/02343) ("Chapman") in view of Ng *et al.* (U.S. Patent Application Publication 2002/0068325) ("Ng") and Gentzsch *et al.* (FEBS Lett 377: 128-130, 1995) ("Gentzsch").

The Examiner alleged that Chapman discloses a recombinant type III AFP HPLC-12 produced in *Saccharomyces cerevisae*, Ng discloses O-linked glycosylation in the ER by pmts, and the Gentzsch discloses pmt 1 and pmt2 deletion mutants. The Examiner alleged it would be

obvious to recombinantly produce the AFP of Chapman in the pmt mutants of Gentzsch given the revelations concerning glycosylation disclosed by Ng.

It is respectfully submitted that Ng discloses pmt mutants as a means to affect protein folding, not as a means to affect protein activity. As the specification states, it was thought in the art that glycosylation of AFPs was required for proteins to function (*see* specification at page 4, lines 7-23). Furthermore, the AFP as disclosed by Chapman is not produced in a glycosylation deficient cell, but yet is a functional protein with high antifreeze activity. Chapman, at page 4, lines 21-37, specifically states that the AFP produced "does not exhibit reduced activity." Accordingly, there was no motivation in the art at the time the present invention was filed to produce an unglycosylated AFP or even suggestion that unglycosylated AFP would be expected to function with any reasonable expectation of success. In fact, no reference indicates a suggestion that glycosylation may in fact reduce the activity of AFP.

Furthermore, no cited reference demonstrates that the pmt1 and pmt2 enzymes glycosylate the AFP of the claimed invention. The Examiner acknowledged that there are six non-redundant pmt proteins in yeast with different substrate specificity. Accordingly, there is no guidance in the art as to which pmt glycosylates the AFP of the claimed invention.

The Examiner further stated that the reference of Van der Laar *et al.* (WO 2002/048382) ("Van der Laar") was not applied in the rejection as it is merely cumulative of Chapman. Van der Laar disclose production of a heterologous yeast from a medium with over 50% ethanol. It is respectfully submitted that Van der Laar fails to overcome the deficiencies of Ng, Chapman, and Gentzsch to render the claimed invention obvious. It is therefore respectfully requested that the Examiner's rejection under 35 U.S.C. § 103(a) be withdrawn.

Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request entry of the amendments, reconsideration, and the timely allowance of the pending claims. A favorable action is awaited. Should an interview be helpful to further prosecution of this application, the Examiner is invited to telephone the undersigned.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for an extension of time

under 37 C.F.R. §1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted
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Transmembrane Topology of Pmt1p, a Member of an Evolutionarily Conserved Family of Protein *O*-Mannosyltransferases*

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The identification of the evolutionarily conserved family of dolichyl-phosphate-D-mannose:protein *O*-mannosyltransferases (Pmts) revealed that protein *O*-mannosylation plays an essential role in a number of physiologically important processes. Strikingly, all members of the Pmt protein family share almost identical hydropathy profiles; a central hydrophilic domain is flanked by amino- and carboxyl-terminal sequences containing several putative transmembrane helices. This pattern is of particular interest because it diverges from structural models of all glycosyltransferases characterized so far. Here, we examine the transmembrane topology of Pmt1p, an integral membrane protein of the endoplasmic reticulum, from *Saccharomyces cerevisiae*. Structural predictions were directly tested by site-directed mutagenesis of endogenous *N*-glycosylation sites, by fusing a topology-sensitive monitor protein domain to carboxyl-terminal truncated versions of the Pmt1 protein and, in addition, by *N*-glycosylation scanning. Based on our results we propose a seven-transmembrane helical model for the yeast Pmt1p mannosyltransferase. The Pmt1p amino terminus faces the cytoplasm, whereas the carboxyl terminus faces the lumen of the endoplasmic reticulum. A large hydrophilic segment that is oriented toward the lumen of the endoplasmic reticulum is flanked by five amino-terminal and two carboxyl-terminal membrane spanning domains. We could demonstrate that this central loop is essential for the function of Pmt1p.

Glycosylation is one of the most elaborate covalent protein modifications known. The carbohydrate chains can be coupled to the protein through either an *N*- or *O*-glycosidic bond. Protein *O*-mannosylation, originally observed in fungi (1), is initiated at the endoplasmic reticulum by protein mannosyltransferases (Pmts)[1] that catalyze the transfer of a mannosyl residue from dolichyl phosphate-activated mannose (Dol-P-Man) to serine or threonine residues of nascent proteins entering the secretory pathway; in the Golgi apparatus additional sugars are added to the *O*-linked mannose with GDP-mannose serving as carbohydrate donor (2, 3). Dol-P-Man-dependent *O*-glycosylation of secreted proteins is a general feature of yeasts and filamentous fungi (4).

The key enzyme of protein *O*-mannosylation, the Dol-P-Man: protein *O*-mannosyltransferase Pmt1p, was purified from *Saccharomyces cerevisiae* following the enzyme activity, and the corresponding gene was cloned (5, 6). Pmt1p is an integral membrane glycoprotein located at the ER (5, 7–9). Based on homology to Pmt1p, a family of seven protein *O*-mannosyltransferases (Pmt1p–Pmt7p) has been identified (10–13). Thus far, protein *O*-mannosyltransferase activity has been demonstrated for Pmt1p, Pmt2p, Pmt3p, Pmt4p, and Pmt6p (13, 14). The individual mannosyltransferases recognize specific protein substrates that might explain the presence of more than one transferase in *S. cerevisiae* (14). Moreover, Pmtp orthologues have been identified from other yeasts (4), from the opportunistic fungal pathogen *Candida albicans* (15), and from *Drosophila melanogaster* (16) suggesting that protein *O*-mannosylation may be common among eucaryotes.

The isolation of *pmt* mutants showed that protein *O*-mannosylation plays a substantial role in a number of physiologically important processes. In the yeast *S. cerevisiae*, protein *O*-mannosylation is an indispensable modification for the maintenance of cell integrity (13). Deletion of the *PMT1* homologue in *C. albicans* results in defects in morphogenesis, a significant loss of virulence, and reduced adherence to host cells (15). In addition, mutations at the *Drosophila PMT1* orthologous locus, *rotated abdomen*, alter muscle structures and the alignment of adult cuticle (16). Despite the functional importance of the evolutionarily conserved Pmtp mannosyltransferases, the initial steps of protein *O*-mannosylation are still very poorly understood.

Pmtp family members are, on average, 50–55% homologous overall with most variation occurring in the length and sequence of amino and carboxyl termini. Most interestingly, all of the Pmts share a nearly identical hydropathy profile, wherein an integral membrane protein with a tripartite structure (amino- and carboxyl-terminal regions, each with several putative transmembrane helices, and a central hydrophilic segment) is predicted (6, 10, 11, 15, 16). Strikingly, this pattern diverges from structural models of other ER glycosyltransferases as well as from the common type II model of glycosyltransferases of the Golgi apparatus.

In the present study we report the mapping of the membrane topology of *S. cerevisiae* Pmt1p using site-directed mutagenesis, carboxyl-terminal reporter fusions, and *N*-glycosylation scanning. These topology-sensitive monitors can distinguish between the lumen of the ER and the cytoplasm. We propose a structural model indicating that Pmt1p spans its cognate membrane seven times. In addition, we demonstrate that a large luminally oriented hydrophilic loop is essential for Pmt1p function.

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[1] The abbreviations used are: Pmt, protein *O*-mannosyltransferase; aa, amino acid; Dol-P-Man, dolichyl-phosphate-D-mannose; Endo H, endoglycosidase H; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GPT, GlcNAc-1-P-transferase; His4C, histidinol dehydrogenase protein domain; TM, transmembrane domain; HA, hemagglutinin epitope; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); oligo, oligonucleotide(s); PCR, polymerase chain reaction.

EXPERIMENTAL PROCEDURES

Yeast Strains

The *S. cerevisiae* strain STY50 (*MATa, his4-401, leu2-3, -112, trp1-1, ura3-52, HOL1-1, suc2::LEU2*) was derived from the strain FC2a (17) by disruption of the *SUC2* gene by homologous recombination. For this purpose, FC2a was transformed with the plasmid pRR8.01 (kindly provided by L. Lehle, University of Regensburg) digested with *Hin*dIII. Yeast shuttle vectors YEp352 (2 μm, *URA3*) (18), pR90 (*PMT1*^{R90}, 2 μm, *URA3*; see below) to pC731 (*PMT1*^{C731}, 2 μm, *URA3*; see below) were transformed into the strain STY50. The *pmt1* deletion strain pmt1Δ (*MATα, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, suc2-Δ9, pmt1::HIS3*) (13) was transformed with the shuttle vectors YEp352, pSB53 (*PMT1*, 2 μm, *URA3*; see below), pSB57 (*PMT1*^{N743A}, 2 μm, *URA3*; see below), pSB60 (*PMT1*^{N390A}, 2 μm, *URA3*; see below), pSB52 (*PMT1*^{G355HA}, 2 μm, *URA3*; see below), pSB62 (*PMT1*^{G355HA} */loop 1*), 2 μm, *URA3*; see below), pSB59 (*PMT1*^{G355HA} */loop 4*), 2 μm, *URA3*; see below), pSB61 (*PMT1*^{G355HA} */loop 6*), 2 μm, *URA3*; see below), pSB73 (*PMT1*, 2 μm, *URA3*; see below) or pSB79 (*PMT1*$^{Δ304-531}$, 2 μm, *URA3*; see below). All yeast transformations were performed following the method of Gietz *et al.* (19). *SUC2* gene disruption was confirmed measuring invertase activity (20).

Plasmid Constructions

Standard procedures were used for all DNA manipulations (21). All cloning and transformations were carried out in *Escherichia coli* host DH5α. PCR fragments were routinely checked by sequence analysis.

PMT1-HIS4C Fusion Plasmids—A 1.26-kilobase pair *Ase*I-*Hin*dIII fragment (bp −343 to +914 of *PMT1*) was subcloned from pDM3 (6) into pBluescript KS+ (Stratagene) digested with *Hin*dIII and *Sma*I resulting in the plasmid pK1A. The *PMT1* coding region from bp +889 to +1065 was amplified by PCR with the primer pairs oligo A11 (5′-actttggacggggatggc-3′) and oligo A12 (5′-gactcgctcgagaccagctggataatt-gtg-3′; *Xho*I site is underlined). The PCR fragment was subcloned as a *Hin*dIII-*Xho*I fragment into pK1A (*Hin*dIII-*Xho*I). From the resulting plasmid, pK1, the *Sca*I-*Xho*I fragment was subcloned into pAΔ7 (digested *Sac*I, *Xho*I) carrying the *SUC2-HIS4C* fusion construct (17) resulting in plasmid pG355. pR90 was constructed by amplifying *PMT1* from bp −137 to +270 with oligo A13 (5′-tgtcgaagaagagtttggcg-3′) and oligo A15 (5′-ttaccgctcgagcctaatgtattgcgaggc-3′), digesting the fragment with *Bgl*II-*Xho*I, and subcloning it into pK1 (digested *Bgl*II and *Xho*I). The *Sac*I-*Xho*I fragment of the resulting plasmid was then subcloned into pAΔ7. The construction of pR157 was analogous to that of pR90 using oligos A13 and A16 (5′-ttaccgctcgagacgtaaagtcatgtacatc-3′). For pV175 oligo A13 and oligo 105 (5′-acgtacgactcgagaacggcaaagcagatagcgctc-3′), for pL221 oligos A13 and A110 (5′-ttaccgctcgagaagcaaggacttgtaagc-3′) were used to amplify *PMT1* from bp −137 to +525 and bp −137 to +659, respectively. The PCR fragments were digested with *Bsh*1365I and *Xho*I and subcloned into pR157 (cut with *Bsh*1365I and *Xho*I). For pS263 to pC731 the following regions of *PMT1* were amplified by PCR: pS263, bp +550 to +789, oligos A19 (5′-cgttacattctgttggacgc-3′) and A111 (5′-ttaccgctcgagggaagacttagtcaaatcc-3′); pF306, bp +550 to +918, oligos A19 and A112 (5′-cattagctcgagaaagaagcttgcgccatcc-3′); pP616, bp +550 to +1848, oligos A19 and A17 (5′-ttaccgctcgagtggtttacctaactgcc-3′); pH655, bp +550 to +1965, oligo A19 and oligo101 (5′-ttaccgctcgaggtgat-gcaaaaacatttgacgttg-3′); pC731, bp +550 to +2193, oligos A19 and A18 (5′-ttaccgctcgagacaattgtagtcccaacc-3′). The PCR fragments were digested with *Pst*I and *Xho*I and subcloned into pL221 cut with the same.

Plasmid pSB52 (PMT1^{G355HA}*)*—A 111-bp *Not*I fragment encoding three copies of the hemagglutinin (HA) epitope was isolated from pAxl2 (22) and subcloned into pGEMEX-1 (Promega). Sequence analysis was used to identify clones with the 5′-sequence of the HA epitope following the *Xho*I site of the vector. The HA epitope sequence (*Xho*I-*Sph*I fragment) was further subcloned from this construct (pSB50) into pG355 (*Xho*I, *Pfl*mI-digested), resulting in pSB52.

Plasmid pSB53 (PMT1)—A carboxyl-terminal fragment of *PMT1* was amplified on genomic DNA using the primer pair oligo A14 (5-tcttgttatggttacagcgg-3′) and oligo 133 (5′-tcactagcatgcggatccaccttcag-caaatg-3′). The PCR-fragment was digested with PflmI and *Sph*I and subcloned into pC731 (cut with PflmI, *Sph*I).

PMT1 Mutants—Deletions or insertions of *N*-glycosylation sites were attained by site-directed mutagenesis using the QuickChange™ Site-directed Mutagenesis Kit from Stratagene. To create pSB57 (*PMT1*^{N743A}) and pSB60 (*PMT1*^{N390A}) the plasmid pSB53 was used as template DNA. The primer pairs oligo 124 (5′-gaagagtacaaagcccaaacct-tgactaaacgt-3′) and oligo 125 (5′-cacgtttagtcaaggtttgggctttgtactcttc-3′) were utilized to make pSB57, oligo 122 (5′-acaacattccaagccctaaccgatg-gtaccaaggtc-3′) and oligo 123 (5′-gaccttggtaccatcggttagggcttggaatgttgt-3′) to make pSB60. pSB52 served as the template DNA to produce pSB62 (*PMT1*^{G355HA} */loop 1*)), pSB59 (*PMT1*^{G355HA} */loop 4*)), and pSB61 (*PMT1*^{G355HA} */loop 6*)). pSB62 was created using the primer pair oligo 138 (5′-cctcctcttgcaaagaacttgtctgctggtaacgcatcgcttggtggg-3′)/oligo 139 (5′-cccaccaagcgatgcgttaccagcagacaagttctttgcaagaggagg-3′), pSB59 the primer pair oligo 117 (5′-gcaagcaaggacttgttagcattggtcgagttgtcagggtac-at-3′)/oligo 118 (5′-atgtaccctgccaactcgaccaatgctaacaagtccttgcttgc-3′), and pSB61 the primer pair oligo 140 (5′-ggcgcaagcaacttttcgcctgaattta-actctacactaaagaac-3′)/oligo 141 (5′-gttctttagtgtagagttaaattcaggcgaaaa-gttgcttgcgcc-3′). To create pSB79 (*PMT1*$^{Δ304-531}$), the plasmid pSB53 was digested with *Sph*I, *Nar*I, and religated. The resulting plasmid (pSB73) was cut with *Hin*dIII and religated, thereby deleting the *PMT1* sequence coding for aa 304 to aa 531.

Computer Analyses

Structural predictions of Pmt1p were made using the programs TMAP (23) and TMPRED. The latter uses an algorithm based on the statistical analysis of TMbase (24). Furthermore, structural models made by Martinsried Institute for Protein Sequences accession number A47716 and SWISS-PROT accession number P33775, were used.

Analysis of the Pmt1-His4C Fusion Proteins

Growth on Histidinol—The strain STY50 was transformed with the plasmids pR90 to pC731. Transformants were selected for the URA3-containing plasmids on SD plates supplemented with the amino acids and bases required at 20–30 mg/liter, lacking uracil and containing 2% glucose. Ura$^+$ transformants were streaked on supplemented minimal medium lacking histidine but containing 6 mM histidinol. The plates were incubated at 30 °C for 3–5 days.

Immunoprecipitation from Whole-cell Extracts—Yeast cells were grown on SD medium to a concentration of 2.0 × 10^7 cells/ml. Cells (50 ml) were harvested and whole-cell extracts prepared as described previously (25). 10 μl of anti-invertase antibody (26) were added to 400 μl of whole-cell extract and incubated for at least 3 h at 4 °C. Thereafter, 400 μl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% sodium deoxycholate, 2% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.25 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 50 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) were added and the extracts dissected. 15 μl of bead volume protein A-Sepharose C-4B beads (Pharmacia) were added, and the incubation was continued for 1 h at 4 °C. The immunoprecipitates were washed five times with 1.4 ml of lysis buffer and once with 1.4 ml of 50 mM potassium phosphate buffer, pH 5.5, 0.02% SDS, protease inhibitors as above. Subsequently, the precipitates were subjected to endoglycosidase H digestion or mock treated.

Preparation of Crude Membranes

Yeast cells were grown on SD medium. At a concentration of 2.0 × 10^7 cells/ml, 20 ml of cells were harvested, washed with 10 ml of 50 mM Tris-HCl, pH 7.5, 0.3 mM $MgCl_2$, and resuspended in 100 μl of the same buffer plus protease inhibitors (see above). An equal volume of glass beads was added, and the cells were lysed by vortexing, for 1 min, four times (with 1-min intervals on ice). The bottom of the tube was punctured and the lysate collected. Cell debris were removed by centrifugation for 5 min at 3,000 rpm at 4 °C. Membranes were collected from the supernatant by centrifugation for 30 min at 20,000 rpm at 4 °C and resuspended in 100 μl of 50 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$.

Isolation of Chitinase

Yeast cells were grown on SD medium to 2.0 × 10^7 cells/ml. Chitinase (Cts1p) was isolated from cell walls as described in Gentzsch and Tanner (14).

Deglycosylation by Endoglycosidase H Digestion

Immunoprecipitates or 5 μl of crude membranes were suspended in 25 μl of Endo H buffer (50 mM potassium phosphate buffer pH 5.5, 0.02% SDS, 0.1 M 2-mercaptoethanol, protease inhibitors as above) and digested with 1–5 units/μl of Endo H for 1 h at 37 °C. Mock samples were incubated without Endo H. The reaction was stopped by adding 10 μl of 5× SDS sample buffer.

Western Blot Analyses

Proteins were fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose (27). Anti-Pmt1p and anti-invertase polyclonal antibodies were used at 1:1,000, anti-Cts1p polyclonal antibody at 1:2,500, and anti-HA monoclonal antibody (16B12; Babco) at 1:5,000

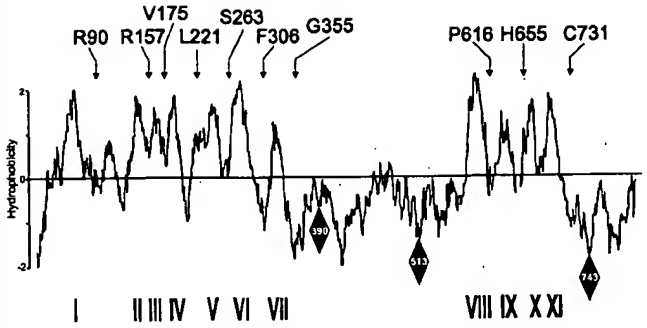

FIG. 1. **Carboxyl-terminal Pmt1-His4C reporter fusion constructs.** The hydropathy profile of Pmt1p using a window of 17 amino acids is shown (60). *Solid diamonds* indicate *N*-glycosylation sites. Potential transmembrane spanning domains are marked by *roman numerals*. The terminal amino acids of the Pmt1 portion in the individual Pmt1-His4C fusion proteins are shown.

dilution. Protein-antibody complexes were visualized by enhanced chemiluminescence using the Amersham Pharmacia Biotech ECL system.

In Vitro Dol-P-Man:Protein O-Mannosyltransferase Assay

5–30 μg of membrane protein were incubated in the *in vitro* assay for Dol-P-Man:protein *O*-mannosyltransferase, as described previously (5). The pentapeptide acetyl-YATAV-NH_2 was used at a final concentration of 3.5 mM.

RESULTS

The Central Hydrophilic Loop and the Carboxyl-terminal End of Pmt1p Are Facing the ER Lumen—S. cerevisiae Pmt1p is a protein of 817 amino acids with three potential *N*-glycosylation sites as follows: two are located in the central hydrophilic loop (aa Asn-390 and Asn-513) and one at the carboxyl-terminal end (aa Asn-743) of the protein (Fig. 1). Treatment with endoglycosidase H (Endo H) reduces the molecular mass of the protein from 92 to 84 kDa (Fig. 2, *lanes 2* and *3*) (5). Considering the fact that Pmt1p resides in the ER (7–9) where only core glycosylation takes place, this difference in molecular mass indicates that all three *N*-glycosylation sequences (N*X*(S/T)) are glycosylated *in vivo*. Since *N*-glycosylation is carried out exclusively on the lumenal side of the ER, these data indicate that the *N*-glycosylation sites are exposed to the ER lumen. To verify this predicted orientation, Pmt1p mutant proteins were constructed wherein the *N*-glycosylation sequons N390LT and N743QT were destroyed individually by changing the asparagines to alanine. The mutant mannosyltransferases were analyzed in the *S. cerevisiae pmt1* deletion strain pmt1Δ. $Pmt1p^{N390A}$ and $Pmt1p^{N743A}$ are functionally active as demonstrated by their *in vitro* mannosyltransferase activity (Table I). Western blot analysis showed that the molecular mass of both mutant proteins decreases from 92 to 89 kDa compared with wild type Pmt1p (Fig. 2, *lanes 3–5*). This is consistent with the loss of one *N*-linked sugar chain, demonstrating that the sequons N390LT and N743QT are *N*-glycosylated *in vivo*. From these results we conclude that the central hydrophilic loop (aa 295–580) and the carboxyl-terminal end (aa 720–817) of Pmt1p are facing the ER lumen.

Carboxyl-terminal Pmt1-His4C Reporter Fusions Reveal the Presence of Seven Membrane Spanning Domains—Resting upon the results of the $Pmt1p^{N390A}$/$Pmt1p^{N743A}$ mutant analyses we used computer based algorithms (see "Experimental Procedures") to propose three structural models of Pmt1p that featured 7, 10, or 11 transmembrane domains. To distinguish between the three models we used fusion constructs of Pmt1p and a truncated version of the His4p protein (His4C) as topology-sensitive reporters. His4C maintains histidinol dehydrogenase activity and is translocated through the ER membrane

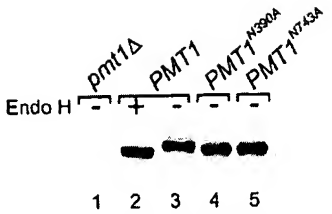

FIG. 2. **Analysis of endogenous *N*-glycosylation mutants.** Crude membranes were isolated from the yeast strains pmt1Δ/YEp352 (pmt1Δ; *lane 1*), pmt1Δ/pSB53 (*PMT1*; *lanes 2* and *3*), pmt1Δ/pSB60 ($PMT1^{N390A}$; *lane 4*), and pmt1Δ/pSB57 ($PMT1^{N743A}$, *lane 5*) and treated with Endo H as indicated. Proteins (25 μg) were resolved on 8% SDS-polyacrylamide gels and analyzed by Western blot using an anti-Pmt1p antibody.

TABLE I
Effects of mutations in N-glycosylation sequons on O-mannosyltransferase activity

PMT1, $PMT1^{N390A}$, and $PMT1^{N743A}$ were individually expressed from the multicopy plasmid YEp352 in the *S. cerevisiae* strain pmt1Δ. Crude membranes were isolated as described under "Experimental Procedures." 5–30 μg of protein were incubated in the *in vitro* mannosyltransferase assay following the transfer of [^{14}C]mannose from Dol-P-Man to the pentapeptide Ac-YATAV-NH_2 (5). Average values of a typical experiment are shown.

Strain	[^{14}C]Mannose transferred *in vitro*
	cpm/mg/min
pmt1Δ	220 ± 99
pmt1Δ/*PMT1*	3500 ± 23
pmt1Δ/$PMT1^{N390A}$	3650 ± 19
pmt1Δ/$PMT1^{N743A}$	3660 ± 7

when fused to a signal sequence (28). Yeast *his4* mutant strains expressing a His4C fusion protein are able to grow on minimal medium containing histidinol when the catalytic domain is present on the cytoplasmic side of the ER membrane. In this case histidinol is metabolized to histidine, resulting in a His^+ phenotype. When the catalytic domain is targeted to the ER lumen histidinol cannot be converted to histidine, resulting in a his^- phenotype. In addition, the protein becomes extensively glycosylated due to the presence of several *N*-glycosylation sites.

We designed a series of fusion proteins consisting of carboxyl-terminal truncated versions of Pmt1p and the His4C protein domain ($Pmt1^{R90}$ to $Pmt1^{C731}$; Fig. 1 and Table II) which allowed us to distinguish between distinct numbers of transmembrane domains as well as their orientation. In addition, the constructs contained a part of the yeast invertase introducing an epitope for immunopurification. The fusion constructs were transformed into a *his4* mutant background (STY50), and the transformants were tested for the ability to grow on selective medium supplemented with histidinol. Furthermore, using a polyclonal antibody directed against an unglycosylated form of invertase (26), the fusion proteins were immunoprecipitated from whole-cell extracts, treated with Endo H, and analyzed by Western blot.

First we wanted to distinguish between an odd *versus* an even number of membrane spanning domains. The number of transmembrane helices dictates the lumenal or cytoplasmic orientation of the amino and carboxyl termini. The 10 transmembrane helical model requires that both termini face the ER lumen. On the other hand, in case of a protein with 7 or 11 transmembrane helices where the carboxyl terminus faces the ER lumen, the amino terminus would face the cytoplasm.

To determine the orientation of the amino terminus the deletion construct $Pmt1^{R90}$ carrying the first putative transmembrane domain (Fig. 1) was analyzed. As shown in Fig. 3, $Pmt1^{R90}$ does not support growth on histidinol indicating that

TABLE II
Pmt1-His4C fusion proteins

Pmt1-His4C fusion proteins ($Pmt1^{R90}$ to $Pmt1^{C731}$) were constructed as described under "Experimental Procedures." The invertase and His4C portion accounts for 122,084 Da of the total molecular mass of each fusion protein and carries 14 potential *N*-glycosylation sites. The mass increases by 35,000 Da when the protein becomes *N*-glycosylated. Growth on histidinol-containing medium and a lack of *N*-glycosylation indicate that the His4C protein domain is facing the cytoplasm, whereas no growth on histidinol and extensive *N*-glycosylation argue for ER luminal orientation.

Pmt1-His4C fusion	Calculated mass	Growth on histidinol	Glycosylation of His4C
	Da		
$Pmt1^{R90}$	132,201	−	+
$Pmt1^{R157}$	139,562	+	−
$Pmt1^{V175}$	141,548	−	+
$Pmt1^{L221}$	146,862	+	−
$Pmt1^{S263}$	151,448	+	−
$Pmt1^{F306}$	156,281	−	+
$Pmt1^{G355}$	161,565	−	+
$Pmt1^{P616}$	191,600	+	−
$Pmt1^{H655}$	196,247	+	−
$Pmt1^{C731}$	205,034	−	+

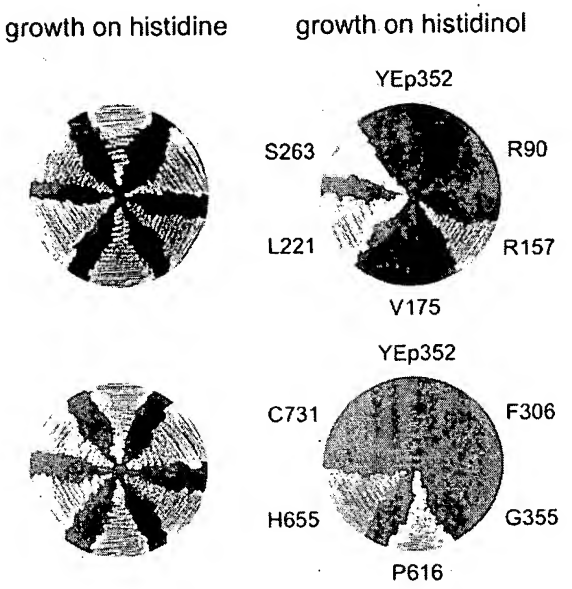

FIG. 3. **Growth phenotypes of Pmt1-His4C fusions.** The yeast strain STY50 was transformed with the plasmid YEp352 or plasmids coding for the Pmt1-His4C fusions $Pmt1^{R90}$ to $Pmt1^{C731}$ (Arg-90 to Cys-731). Transformants were streaked on selective media supplemented with histidine (*left panels*) or histidinol (*right panels*) and incubated for 3–5 days at 30 °C.

His4C is facing the lumen of the ER. This is validated by the fact that the fusion protein is highly glycosylated *in vivo* (Fig. 4, *lanes 3* and *4*). $Pmt1^{R90}$ shows an apparent molecular mass of 167 kDa which decreases after Endo H treatment to 132 kDa. This is in agreement with the calculated mass of the unglycosylated protein (Table II). These results indicate that the amino terminus of this construct resides on the cytoplasmic side of the ER membrane. A very minor fraction of $Pmt1^{R90}$, which varied in its abundance from experiment to experiment, is not glycosylated (Fig. 4, *lane 3*). We presume that this fraction is either oriented with the His4C domain in the cytoplasm or not translocated to the ER at all, remaining misfolded in the cytoplasm. The latter possibility would explain why this protein does not provide any growth on histidinol (Fig. 3).

To confirm that the carboxyl terminus faces the ER lumen we used the reporter fusion $Pmt1^{C731}$, which contains all the potential transmembrane domains. $Pmt1^{C731}$ produces a His^-

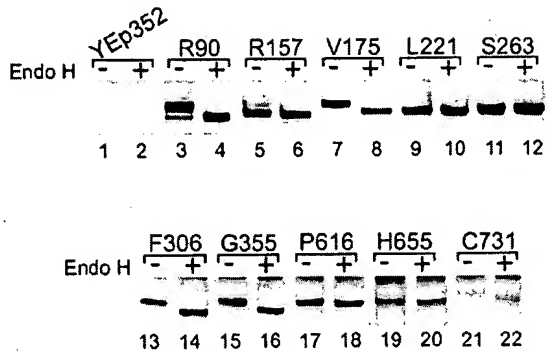

FIG. 4. **Analysis of the *N*- glycosylation state of the Pmt1-His4C fusion proteins.** The Pmt1-His4C fusion proteins $Pmt1^{R90}$ to $Pmt1^{C731}$ were immunoprecipitated from whole-cell extracts made from STY50 transformed with the plasmid YEp352 (*lanes 1* and *2*) or plasmids pR90 to pC731 (*lanes 3–22*) using a polyclonal anti-invertase antibody. Immunoprecipitates were treated with Endo H as indicated and separated on 7% SDS-polyacrylamide gels. Western analysis was performed using an anti-invertase antibody.

phenotype (Fig. 3) and is extensively glycosylated (Fig. 4, *lanes 21* and *22*) indicating that the His4C domain is oriented toward the ER lumen. Since the amino and carboxyl termini are located on different sides of the ER membrane we exclude the 10 transmembrane helical model. Furthermore, $Pmt1^{G355}$ shows the same phenotype as $Pmt1^{C731}$ confirming the lumenal orientation of the hydrophilic central part of Pmt1p (Fig. 3 and Fig. 4, *lanes 15* and *16*). Taken together, these results indicate the presence of 7 or 11 transmembrane domains with an odd number of helices between the amino terminus and the middle loop and an even number between the latter and the carboxyl terminus of Pmt1p.

Next, we wished to discriminate between the 7 and the 11 transmembrane helical model. In the fusion protein $Pmt1^{R157}$ the His4C domain is oriented toward the cytoplasm as indicated by the growth of the transformants on medium supplemented with histidinol (Fig. 3). Accordingly, the mobility of the protein on SDS-PAGE is not affected by Endo H treatment showing that His4C is not glycosylated (Fig. 4, *lanes 5* and *6*). Evidently, the catalytic His4C domains of $Pmt1^{R90}$ and $Pmt1^{R157}$ are on different sides of the ER membrane. The fusion $Pmt1^{V175}$ is facing the ER lumen (Fig. 3 and Fig. 4, *lanes 7* and *8*) and $Pmt1^{L221}$ the cytoplasmic side of the membrane (Fig. 3 and Fig. 4, *lanes 9* and *10*). These data confirm that the predicted transmembrane domains TM I, TM II, TM III, and TM IV (Fig. 1) are spanning the membrane *in vivo*. Unexpectedly, the catalytic domain of the fusion $Pmt1^{S263}$ is also facing the cytoplasm (Fig. 3 and Fig. 4, *lanes 11* and *12*) implying that TM V does not cross the membrane. The His4C domains of both fusion proteins $Pmt1^{P306}$ and $Pmt1^{G355}$ are located in the ER lumen (Fig. 3 and Fig. 4, *lanes 13–16*) indicating that TM VI, but not TM VII (Fig. 1), traverses the membrane *in vivo*. These data argue for the presence of five membrane spanning domains in the amino-terminal half of Pmt1p.

How many transmembrane helices are present between the hydrophilic middle part and the carboxyl terminus? To answer this question the fusion proteins $Pmt1^{P616}$ and $Pmt1^{H655}$ were analyzed. $Pmt1^{P616}$ is oriented with the His4C catalytic domain on the cytoplasmic side of the ER membrane (Fig. 3). Therefore, TM VIII (Fig. 1) does span the membrane *in vivo*. A minor increase in the mobility of the protein after Endo H treatment is due to the removal of two *N*-linked carbohydrate chains at positions Asn-390 and Asn-513 in the Pmt1p portion of the fusion (Fig. 4, *lanes 17* and *18*). A very similar result was obtained for the fusion protein $Pmt1^{H655}$ (Fig. 3 and Fig. 4, *lanes 19* and *20*) showing that TM IX does not cross the mem-

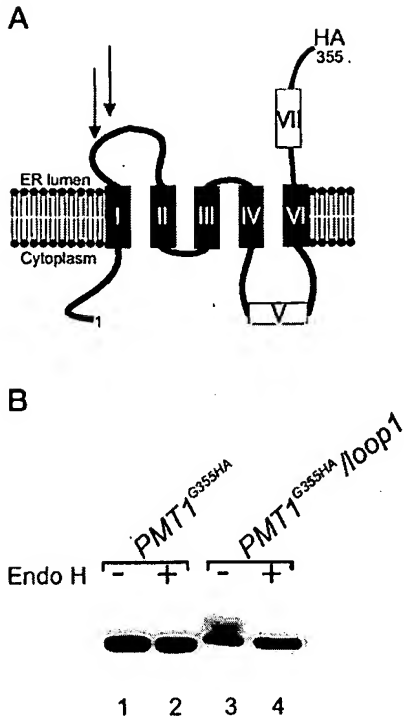

FIG. 5. **Transmembrane topology of Pmt1^{355HA}.** *A*, predicted transmembrane topology of Pmt1^{355HA} deduced from the Pmt1-His4C fusion experiments. *Arrows* indicate the *N*-glycosylation sites inserted in the construct Pmt1^{355HA}/loop 1 (see also Table III). *Roman numerals* mark the putative transmembrane spanning domains. *HA*, hemagglutinin epitope. *B*, crude membranes (25 μg of protein), isolated from the yeast strains pmt1Δ/pSB52 (*PMT1^{355HA}*; *lanes 1* and *2*) and pmt1Δ/pSB62 (*PMT^{355HA}/loop 1*; *lanes 3* and *4*), were resolved on 10% SDS-polyacrylamide gels after incubation with Endo H. Western analysis was performed using a monoclonal anti-HA antibody.

brane. These data, in combination with the prediction of an odd number of transmembrane helices between the central hydrophilic loop and the carboxyl-terminal end, demonstrate the presence of two transmembrane helices in the carboxyl-terminal half of Pmt1p.

Glycosylation Scanning Mutagenesis Substantiates the Prediction of Five Transmembrane Helices in the Amino-terminal Region of Pmt1p—Our results of the His4C fusion experiments favor the 7 transmembrane helical model with the helices TM I, TM II, TM III, TM IV, and TM VI but not TM V and TM VII (Fig. 1) serving as membrane spanning domains in the amino-terminal half of the protein. On the other hand, computer programs analyzing the Pmt1p hydropathy profile (see "Experimental Procedures") predicted TM V to be a transmembrane helix. To verify that TM V does not span the membrane *in vivo* we used *N*-glycosylation scanning mutagenesis. Since the high molecular weight of the native Pmt1p, its own *N*-glycosylation, and its residence in the ER complicate this kind of analysis, we circumvented these problems by constructing a truncated version of Pmt1p, Pmt1^{G355HA} (Fig. 5*A*), where the carboxyl-terminal amino acids 356 to 817 are substituted with the hemagglutinin (HA) epitope (29). This protein has a calculated mass of 45.3 kDa, no endogenous *N*-glycosylation sites, and is immunologically detectable. On SDS-PAGE Pmt1^{G355HA} migrates at 43.5 kDa (Figs. 5*B* and 6*C*, *lanes 1* and *2*). In addition, we detected a second minor species with an apparent mass of 42 kDa varying in its intensity in different experiments. This species is assumed to be either a degradation product or a modified version of the 43.5-kDa protein. The carboxyl terminus of Pmt1^{G355HA} is facing the ER lumen (data not shown) and thus is consistent with the orientation of the central hydrophilic loop (aa 295–580) of the Pmt1p protein. Therefore, we concluded that this protein reflects the transmembrane topology of the native Pmt1p protein.

TABLE III
N-glycosylation sequons introduced in Pmt1^{G355HA}

Two *N*-glycosylation sequons, underlined below, were introduced between TM I and TM II (Pmt1^{355HA}/loop 1), TM IV and TM V (Pmt1^{355HA}/loop 4), and TM VI and TM VII (Pmt1^{355HA}/loop 6) by site-directed mutagenesis as described under "Experimental Procedures."

Construct	Amino acids exchanged	*N*-Glycosylation sequons inserted
		101
Pmt1^{355HA}/loop 1	M104N, Y106S, V109N	L AKNLSAGNASLGG
		208
Pmt1^{355HA}/loop 4	L214T, Y217S	M YPANSTNASKSLL
		302
Pmt1^{355HA}/loop 6	F305N, R311N	G ASNFSPEFNSTLK

To confirm that the amino terminus faces the cytoplasm, we used Pmt1^{G355HA} and introduced two *N*-glycosylation sequons into the loop region between the transmembrane helices TM I and TM II by site-directed mutagenesis (Fig. 5*A* and Table III). The *N*-glycosylation sites were placed at least 12 amino acids away from adjacent membrane helices to ensure they could become *N*-glycosylated (30). The resulting construct Pmt1^{G355HA}/loop 1 was expressed from a high copy 2-μm plasmid in the *pmt1* mutant strain pmt1Δ. A crude membrane fraction was isolated and treated with Endo H to verify *N*-glycosylation. The proteins were then analyzed by Western blot using a monoclonal antibody directed against the HA epitope. Our data show that the loop region between TM I and TM II is *N*-glycosylated *in vivo*. Core-glycosylated species of Pmt1^{G355HA}/loop 1 were detected in addition to the unglycosylated protein (Fig. 5*B*, *lanes 3* and *4*). It is likely that this partial glycosylation is due to varied numbers of *N*-glycosylation sites being used. Similar effects were observed when Pmt1p was expressed from a high copy 2-μm plasmid.[2] From these results we conclude that the loop region between TM I and TM II is located in the ER lumen and, consequently, that the amino terminus of Pmt1^{G355HA} is oriented toward the cytoplasm, confirming the results obtained by His4C reporter fusions.

To answer the question as to whether TM V is used as a transmembrane span *in vivo*, we independently introduced two *N*-glycosylation consensus sequences in the loop regions between TM IV and TM V (Pmt1^{G355HA}/loop 4; Table III) and between TM VI and TM VII (Pmt1^{G355HA}/loop 6; Table III). Considering the odd number of transmembrane helices predicted to form between the amino terminus and the hydrophilic middle region, it may be expected that either both TM V and TM VII serve as transmembrane spans or neither of them. In the first case loop 4 and loop 6 would be localized in the cytoplasm (Fig. 6*A*); in the second case loop 4 and loop 6 had to be on opposite sides of the membrane wherein loop 6 is facing the ER lumen (Fig. 6*B*). The mutant proteins Pmt1^{G355HA}/loop 4 and Pmt1^{G355HA}/loop 6 were expressed in the strain pmt1Δ. To examine the state of glycosylation crude membranes were isolated, treated with Endo H, and analyzed by Western blot using a monoclonal anti-HA antibody. Fig. 6*C* shows that Pmt1^{G355HA}/loop 6 (*lanes 5* and *6*) but not Pmt1^{G355HA}/loop 4 (*lanes 3* and *4*) is glycosylated *in vivo* providing further evidence that TM V does not serve as a membrane spanning helix. These results demonstrate the presence of only five transmembrane spanning domains in the amino-terminal half of Pmt1p.

The Central Hydrophilic Loop Is Crucial to Pmt1p Function—Summarizing, our data ascertain five transmembrane

[2] M. Gentzsch, personal communication.

spans between the amino terminus and a large central hydrophilic loop region which is facing the ER lumen and two membrane spanning domains between the latter and the carboxyl terminus. The luminally oriented middle loop (aa 295–580) is the largest hydrophilic segment in Pmt1p. Since it is almost certain that the transfer of the mannose from Dol-P-Man to proteins occurs in the ER lumen (31, 32), this loop might be essential for Pmt1p function. To test this, we examined whether the large hydrophilic loop is crucial for Pmt1p enzymatic activity.

We created a mutant version of Pmt1p ($Pmt1^{\Delta 304-531}$; see "Experimental Procedures") that lacks the amino acid residues 304–531 including the *N*-glycosylation sequons Asn-390 and Asn-513 (Fig. 1). $Pmt1^{\Delta 304-531}$ was analyzed in a *pmt1* mutant background. On SDS-PAGE $Pmt1^{\Delta 304-531}$ shows an apparent mass of 64 kDa (Fig. 7*A*, *lane 4*), being similar to the predicted size of 66.9 kDa. Endo H treatment reduces the mass to 62 kDa (Fig. 7*A*, *lane 5*). This decrease in mass of 2 kDa indicates that the only *N*-glycosylation site (Asn-743; see Fig. 1) present in $Pmt1^{\Delta 304-531}$ bears one *N*-linked core carbohydrate chain. From these results we conclude that (i) $Pmt1^{\Delta 304-531}$ resides in the ER membrane and (ii) $Pmt1^{\Delta 304-531}$ mirrors the membrane topology of native Pmt1p.

To test whether $Pmt1^{\Delta 304-531}$ still has mannosyltransferase activity, we analyzed the *in vivo* glycosylation status of the highly *O*-mannosylated protein chitinase (Cts1p; see Ref. 33) in a yeast *pmt1* mutant expressing $Pmt1^{\Delta 304-531}$. Confirming previous results (14) we found that in the *pmt1* deletion strain Cts1p is less glycosylated as compared with a strain where Pmt1p is present (Fig. 7*B*, *lanes 1, 2,* and *4*). The mutant protein $Pmt1^{\Delta 304-531}$ does not repeal the underglycosylation of Cts1p (Fig. 7*B*, *lane 3*). Furthermore, $Pmt1^{\Delta 304-531}$ did not show significant *in vitro* mannosyltransferase activity (data not shown). Since the amounts of $Pmt1^{\Delta 304-531}$ and native Pmt1p protein are very similar in the strain pmt1Δ (Fig. 7*A*, *lanes 2* and *4*), these data definitively prove that the luminally oriented hydrophilic loop is essential for Pmt1p function.

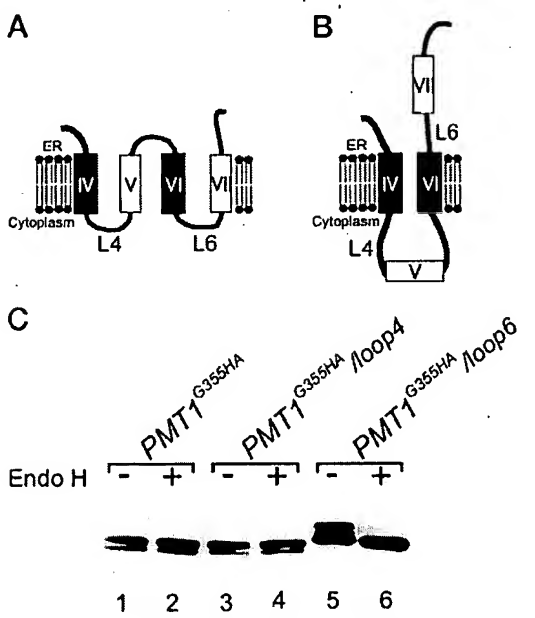

FIG. 6. **Analysis of amino-terminal transmembrane helices.** *A* and *B*, possible arrangements of the transmembrane helices TM IV to TM VII. Putative membrane spans are marked in *roman numerals*. *L4*, loop 4; *L6*, loop 6. *C*, Western analysis of crude membranes isolated from the yeast strains pmt1Δ/pSB52 ($PMT1^{355HA}$; *lanes 1* and *2*), pmt1Δ/pSB59 ($PMT1^{355HA}$/*loop 4*; *lanes 3* and *4*), and pmt1Δ/pSB61 ($PMT1^{355HA}$/*loop 6*; *lanes 5* and *6*) was performed using a monoclonal anti-HA antibody. Membranes (25 μg of protein) were treated with Endo H as indicated.

DISCUSSION

In this study, we present the first analysis of the transmembrane topology of a Pmt-mannosyltransferase, an enzyme crucial to initiating protein *O*-mannosylation at the ER. Our data provide strong genetic and biochemical evidence for a seven-transmembrane helical model, summarized in Fig. 8. The Pmt1p amino terminus faces the cytoplasm, the carboxyl terminus the ER lumen. A large hydrophilic region, located in the ER lumen, is separated from the amino terminus by five and from the carboxyl terminus by two membrane spanning domains. By using deletion mutagenesis we show that the ER luminally oriented central loop is crucial for mannosyltransferase activity.

The successful use of heterologous protein fusions as topology-sensitive monitors (17, 34) encouraged us to use His4C as a reporter of the topological location which discrete portions of Pmt1p acquire in the membrane. The results obtained with Pmt1-His4C fusions are supported by several other lines of evidence as follows. (i) *N*-glycosylation scanning demonstrates that TM I integrates into the membrane with its carboxyl-terminal region reaching into the ER lumen. As a consequence the Pmt1p amino terminus is cytoplasmic. In agreement with these data TM I shows the features of a "type II signal anchor sequence" (reviewed in Ref. 35); TM I (aa 51–70) is amino-terminally flanked by three positive (Arg-42, Lys-48, and Lys-50) and carboxyl-terminally by three negative (Asp-72, Asp-77, and Glu-78) charges. (ii) Mutation of the endogenous Pmt1p *N*-glycosylation sequons N390LT and N743QT demonstrates that the central hydrophilic loop and the carboxyl terminus of Pmt1p are oriented toward the ER lumen. Earlier results resting upon Endo H digestion of Pmt1p isolated from yeast and heterologous expression of Pmt1p in *E. coli* already suggested that all three *N*-glycosylation sites (Asn-390, Asn-513, and Asn-743) are used in the yeast Pmt1p protein (5, 6). (iii) Our *N*-glycosylation scanning data corroborates the prediction that

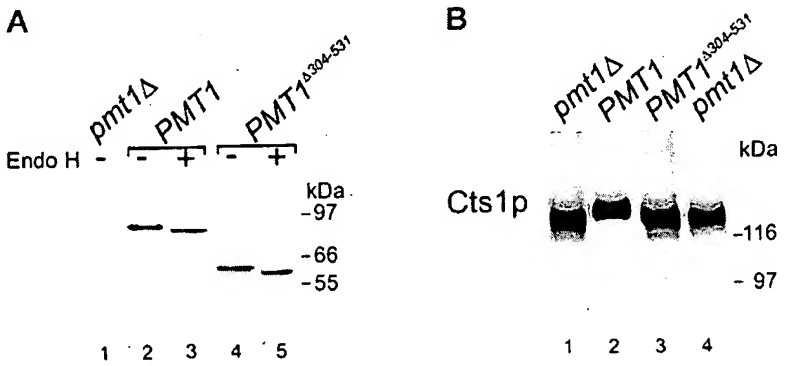

FIG. 7. **The luminally oriented hydrophilic loop is crucial for Pmt1p function.** *A*, crude membranes (25 μg of protein) isolated from the yeast strains pmt1Δ/YEp352 (*pmt1Δ*; *lane 1*), pmt1Δ/pSB73 (*PMT1*; *lanes 2* and *3*), and pmt1Δ/pSB79 ($PMT1^{\Delta 304-531}$; *lanes 4* and *5*) were resolved on a 10% SDS-polyacrylamide gel and analyzed by Western blot using an anti-Pmt1p antibody. Membranes were treated with Endo H as indicated. *B*, chitinase (Cts1p) was isolated from the yeast strains pmt1Δ/YEp352 (*pmt1Δ*; *lanes 1* and *4*), pmt1Δ/pSB73 (*PMT1*; *lane 2*), and pmt1Δ/pSB79 ($PMT1^{\Delta 304-531}$; *lanes 3* and *5*) and analyzed by Western blot as described by Gentzsch and Tanner (14).

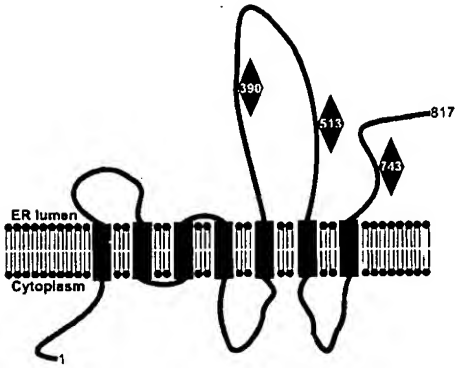

FIG. 8. **Predicted seven-transmembrane helical structure of yeast Pmt1p.** The amino and carboxyl termini face the cytoplasm and the ER lumen, respectively. The central hydrophilic loop is oriented toward the lumen of the ER and is flanked by five amino-terminal and two carboxyl-terminal membrane spanning domains. *N*-glycosylation sites are marked with *solid diamonds*.

hydrophobic helix TM V does not span the membrane. TM V (aa 235–256) is flanked on both sides by positively charged loops and thus might adopt a "leave-one-out" topology as observed by Gafvelin and von Heijne (36) for *E. coli* inner membrane proteins. (iv) The majority of the loop regions between the seven transmembrane helices follow the "positive-inside rule" which states that positively charged residues are often found flanking hydrophobic transmembrane segments on the cytosolic side of the membrane (reviewed in Ref. 37).

The complex organization of yeast Pmt1p contrasts the structure of the mammalian UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases that initiate mucin-type *O*-glycosylation at the Golgi apparatus of higher eucaryotes (38). The GalNAc-transferases show small cytosolic amino-terminal domains, single transmembrane segments, lumenal stem regions, and large carboxyl-terminal lumenal domains responsible for catalysis. This type II model structure is common to glycosyltransferases of the Golgi apparatus, for example the yeast mannosyltransferases Mnt1/Kre2p (39, 40) or Mnn1 (41).

ER resident glycosyltransferases showing multiple putative transmembrane helices were found in *S. cerevisiae* as well as in higher eucaryotes. These include the glycosyltransferases involved in the synthesis of the lipid oligosaccharide precursor for *N*-glycans (3, 42) and the glycosylphosphatidylinositol (GPI) anchor (3, 43). The topological organization of these transferases is not well characterized, but for the ones investigated so far the putative transmembrane topology does not resemble the structure of Pmt1p. A scarce example where the structure-function relationship has been examined in detail is the hamster UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase (GPT) (44, 45). This enzyme initiates *N*-linked glycosylation by catalyzing the synthesis of GlcNAc-P-P-dolichol. Lehrman and coworkers (42, 46) demonstrated that GPT is a multimeric enzyme with multiple, most likely 10 transmembrane spans. The largest hydrophilic segment, located between TM 9 and TM 10, is facing the cytoplasm. This loop region most likely bears the catalytic site consistent with the fact that GlcNAc-P-P-dolichol is synthesized on the cytoplasmic side of the ER. GPT seems to be highly conserved between higher eucaryotes and yeast since the human GlcNAc-1-P transferase complements a *S. cerevisiae alg7* (asparagine linked glycosylation) mutant defective in GPT activity (47). Recently, several ER-located glycosyltransferases have been identified which, like Pmt1p, use dolichol phosphate-activated sugars as donor substrates. These include Alg3p (48), Alg6p (49), Alg8p (50), Alg9p (51), and Alg10p (52) from *S. cerevisiae* which participate in the assembly of the dolichol pyrophosphate-linked oligosaccharide at the lumenal side of the ER, the human PIG-B protein (53) required for GPI anchor synthesis, and its functional homologue Gpi10p from yeast (54). Computer analyses predict the presence of several transmembrane domains in the Dol-P-sugar-utilizing transferases, but only in the case of PIG-B has the membrane topology been investigated. Takahashi *et al.* (53) provided evidence that despite its hydrophobic nature PIG-B shows the topological structure of a type II membrane protein. PIG-B consists of a short amino-terminal cytoplasmic segment, a transmembrane domain, and a large carboxyl-terminal region facing the ER lumen. The discrepancy between the putative and experimentally determined structure of PIG-B demonstrates how cautious computer-based analyses should be interpreted and how important it is to obtain direct structural information.

Our analyses elucidate an elaborate structure for yeast Pmt1p with seven transmembrane domains and a number of loop regions. The large hydrophilic central loop (aa 295–580) is essential for Pmt1p activity, suggesting that the catalytic site is facing the ER lumen. This is in good agreement with previous data showing that Dol-P-Man is used as donor on the lumenal side of the ER membrane for the mannosylation of the *N*-glycan precursor intermediates, for the synthesis of GPI anchors as well as for protein *O*-mannosylation (55). Like for Pmt1p the catalytic domain of PIG-B is also facing the ER lumen since deletion of the cytoplasmic domain does not abolish enzymatic activity (53).

Since protein *O*-mannosylation is an essential modification in yeast (13), Pmt1p may be subject to stringent regulation. Thus, it is possible that some regions are involved in catalysis, whereas others could interact with various proteins or regulators. This assumption is sustained by the observation that Pmt1p interacts with Pmt2p *in vivo,* and the formation of this complex is required for maximum transferase activity (56). Interactions with different regulators are also conceivable and even suggested by the finding that Pmt proteins possess three highly conserved phosphorylation sites for protein kinase C (S*X*(R/K) and T*X*(R/K)) and *pmt* mutants display phenotypes similar to those observed in protein kinase C mutants (13, 57). In addition, *S. cerevisiae* and *C. albicans* Pmt1p can be activated by phospholipids *in vitro* (58).[3] That Pmt *O*-mannosyltransferases are the subject of various regulation mechanisms is emphasized by the fact that yeast *PMT1–PMT6* are also transcriptionally regulated during the cell cycle[3] and during diauxic shift from fermentation to respiration (59).

Future work will be necessary to learn more about the mechanism how Pmts transfer mannose from Dol-P-Man to specific acceptor proteins at the endoplasmic reticulum. This study provides the basis for the identification and characterization of structural and functional important domains of Pmt *O*-mannosyltransferases.

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